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The research covered by this contract investigates the utility of oligodeoxynucleotides (hereinafter called oligonucleotides or ODNs) as antileishmanial agents. The sequence of these ODNs is antisense to the spliced leader sequence of certain mRNA in the Leishmania sp. The scope of the work comprises the exploration of structural modifications that should increase the potency of these ODNs as antiparasitic agents. These modifications include those that improve the hybrid strength (receptor affinity), stability to degradation, and cell penetration of the oligonucleotides.

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I. Introduction

The research covered by this contract investigates the utility of oligodeoxynucleotides (hereinafter called oligonucleotides or ODNs) as antileishmanial agents. The sequence of these ODNs is antisense to the spliced leader sequence of certain mRNA in the *Leishmania* sp. The scope of the work comprises the exploration of structural modifications that should increase the potency of these ODNs as antiparasitic agents. These modifications include those that improve the hybrid strength (receptor affinity), stability to degradation, and cell penetration of the oligonucleotides.

II. Progress Toward Technical Objectives of the Contract

A. Aim 1: Establish an *in vitro* amastigote/macrophage system with *L. mexicana amazonensis* and *L. donovani*.

Before the initiation of this project, an agreement was reached with the contracting officer that all in vitro screening of oligonucletides for anti-leshmanial acitivity would be conducted in the laboratory of Dr. William Hanson, Department of Parisitology, University of Georgia. Compounds were sent in batches to Dr. Hanson, where they were tested at a concentiration of 30 μM for their ability to inhibit multiplication of *L. donovani* promastigotes. Each oligonucleotide was studied in 4 replicate cultures, and results were compared to untreated controls.

Table I. Unmodified Oligonucleotides to determine best target.

Sequence of *L. enriettii* spliced leader RNA:
5′ AAC GCU AUA UAA GUA UCA GUU UCU GUA CUU UAU UG

Test oligonucleotides

MPC #	SEQUENCE	% inhib at 30 μM
ANTISENSE		
LE001	CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT	8
LE002	ACT GAT ACT TAT ATA GCG TT	5
LE003	AT ACT TAT ATA GCG TT	18
LE004	T TAT ATA GCG TT	21
LE005	CAA TAA AGT ACA	12
SENSE		
LE501	AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG	-1
LE502	AAC GCT ATA TAA GTA TCA GT	-5
LE503	AAC GCT ATA TAA GTA T	20
LE504	AAC GCT ATA TAA	32
LE505	T GTA CTT TAT TG	14

The results of all these studies are tabulated in the final section of the report. For the sake of brevity, single letter abbreviations are used for the modifications to the oligonucleotides. These modifications are given in the table footnotes. Additionally, all compounds are referred to by their MicroProbe Corp identification number. In this

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number, the first letters refer to the system to which the sequence is antisense (LE for leishmania), the number is a code number, and the suffix letters refer to the chemical modification.

B. Aim 2: Determine the best chemical modifications to ODNs for increasing their potency in cell culture using 3'-, 5'-, and/or internal base addition of moieties that modify either physicochemical or hybridization properties of the ODN.

1. Determination of best antisense sequence

The results of testing various sequences surronding the conserved spliced leader of Leishmania mRNA are given in Table I. No significant inhibition of Leishmania growth was seen with any of these oligonucleotides. These ODNs are prone to rapid degradation without endcapping reagents. We proceeded, therefore, to prepare the modified derivatives described below to overcome the stability and cell uptake issues. Our previous work in the Phase I of this project had shown that in vitro translation inhibition could be obtained with antisense oligos directed against portions of this leader sequence, and both a long (the LE001 sequence) and a short (the LE 002 sequence) were used. In addition, the sense sequences were used as controls initially. In many experiments, no controls were used, with the anticipation of following up on active compounds with appropriate controls.

2. Synthesis of New 5'-Modification Reagents

One new acridine "tail" was prepared for 5'-modification. This is 9-(5-hydroxypentyl)-acridine, which, when attached to an ODN via a phosphate ester, should provide a stable and nonmetabolizable modification, designed to enhance the strength of binding of the ODN to the mRNA.[1,2] This modification is noted in the code names in the tables as the "S" modification.

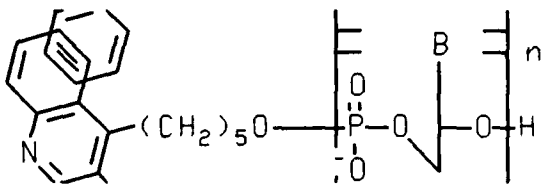


Figure 1. 5'-Acridine Endcapping Modification.

A modification with which we have had considerable success in using in other studies of antisense and antigene antiviral agents is the placement of a cholesterol moiety on either the 3'- or 5'-end of the oligonucleotide. We feel that this reagent enhances the ability of the oligo to penetrate the membrane of cells, a finding seen in many cases. In addiion, it stabilizes the oligo to degradation, as do the other endcappiing reagents. Table III shows the effect of several of these groups on the activity of the oligos.

We have used a 5'-aminohexyl endcapping reagent, designated "Z", as a standard endcap; it is also included in many of the test compounds.

3. Synthesis of New 3'-Modification Reagents

Table II. Effect of small endcapping groups on activity.

ANTISENSE

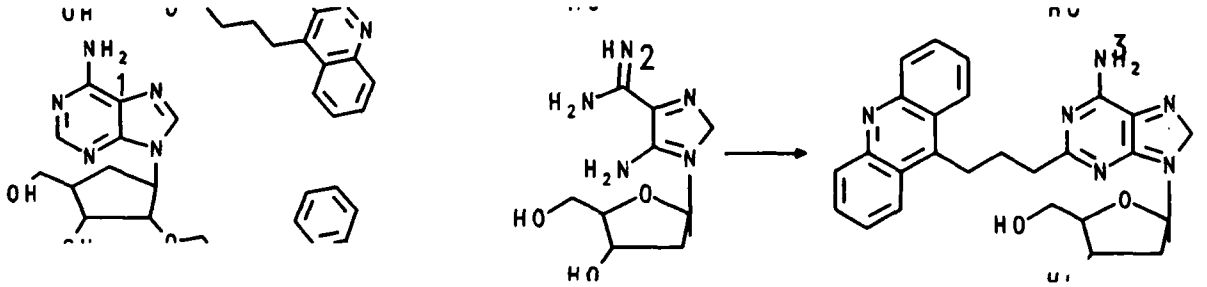
LE001	ZV	CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT	37
LE002	ZV	ACT GAT ACT TAT ATA GCG TT	-4
LE003	ZV	AT ACT TAT ATA GCG TT	-4
LE004	ZV	T TAT ATA GCG TT	43
LE005	ZV	CAA TAA AGT ACA	18

SENSE

LE501	ZV	AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG	7
LE502	ZV	AAC GCT ATA TAA GTA TCA GT	14
LE503	ZV	AAC GCT ATA TAA GTA T	6
LE504	ZV	AAC GCT ATA TAA	49
LE505	ZV	T GTA CTT TAT TG	32

Z modification: aminohexyl tail on the 5'-end
V modification: aminohexyl tail on the 3'-end.

The 3'-position of ODNs was modified by a new method, developed by us, that differs somewhat from that described in the proposal. We have prepared glass bead supports for ODN synthesis on the automated synthesizer which contain a cholesterol or acridine moiety already attached.[3]



The antisense 35-mer with the ZV substitution showed approximately 37% inhibition at the dose tested (30 μM), while the sense control showed essentially no effect. Remarkably, the shortest oligomer tested, the antisense 12-mer LE004, gave 43% inhibition and its sense counterpart gave 49% inhibition. In light of the highly variable nature of the test results, however, this amount of inhibition at this concentration was deemed not significant.

4. Novel Lipophilic Moieties Attached to the Bases

We have prepared a novel derivative of 2'-deoxyuridine with a dodecyl group attached to the 5-position of the pyrimidine base. This modification does not interfere with base pairing, but will modify the overall lipophilicity, and possibly the cell-penetrating power, of the oligonucleotides. One or more of these can be incorporated

into each oligonucleotide, depending on the number of T's in it. Table IV shows the results obtained from 6 different oligos with this modification (designated 3 in the table) in various places, and in numbers of from 1 to 3 bases in the test oligos.

C. Intercalating Agents Tethered to Internal Bases in the ODNs

Molecular modeling experiments conducted here indicate that one of the best places to tether the intercalator is to a base within the ODN. We prepared 5-[5-(9-acridinyl)pentyl]-2'-deoxyuridine for incorporation into antileishmanial ODNs. We have hypothesized that these novel agents should exhibit significantly enhanced hybrid stability and greater in vitro potency in the screening program.

In addition, two new classes of intercalator-tethered nucleosides, shown by structures **1** and **3**, were prepared and incoporated into oligonucleotides for testing. A novel and versatile synthesis of 2-substituted adenosines beginning with compound **2** was developed, and nucleosides **1** and **3** were prepared and incorporated into ODNs for this program. These ODNs did not show inhibition of the Leishmania, as shown in Table IV.

Table III. ODNs with 3'- and/or 5'-cholesterol and acridine endcaps.

LE001QX	3'-$(CH_2)_{16}$-OH; 5'-Cholesterol	-77
LE002QX	3'-$(CH_2)_{16}$-OH; 5'-Cholesterol	-222
LE001SX	3'-Acridine; 5'-Cholesterol	-179
LE002SX	3'-Acridine; 5'-Cholesterol	-10
LE001.01H	3'-Cholesterol; 5'-$(CH_2)_{15}CH_3$	17
LE002.01H	3'-Cholesterol; 5'-$(CH_2)_{15}CH_3$	-14
LE001.01Q	3'-$(CH_2)_{16}$-OH; 5'-$(CH_2)_{15}CH_3$	-35
LE002.01Q	3'-$(CH_2)_{16}$-OH; 5'-$(CH_2)_{15}CH_3$	-32
LE001.01S	3'-Acridine; 5'-$(CH_2)_{15}CH_3$	-27
LE002.01S	3'-Acridine; 5'-$(CH_2)_{15}CH_3$	7

H suffix signifies 3'-cholesterol tail
Q suffix signifies 3'-hexadecanol tail
S suffix signifies 3'-acridine tail
X suffix signifies 5'-cholesterol tail
.01 suffix indicates experimental modification. In these cases it is 5'-hexadecane.

ODNConc (mg/mL)	Conc (mM)	
LE 001HX	3'-cholesterol 5'-cholesterol	8
LE 002HX	3'-cholesterol 5'-cholesterol	-75
LE 001HY	3'-cholesterol 5'-cholesterol	32
LE 002HY	3'-cholesterol 5'-acridine	27
LE 001QY	3'-hexadecanol 5'-acridine	16
LE 002QY	3'-hexadecanol 5'-acridine	-71
LE 001SY	3'-acridine 5'-acridine	-2
LE 002SY	3'-acridine 5'-acridine	ND
LE 001.01J	3'-hexanol	27
LE 501.01J	3'-hexanol	53

H suffix signifies 3'-cholesterol tail
J suffix signifies 3'-hexanol tail
Q suffix signifies 3'-hexadecanol tail
S suffix signifies 3'-acridine tail
X suffix signifies 5'-cholesterol tail
Y suffix signifies 5'-acridine tail
.01 suffix indicates experimental modification. In these cases it is C12 dU.

D. Investigate chemical functional groups that add to the ODN the ability to either catalytically cleave or covalently modify the target nucleic acid.

When the Phase II proposal for this work was submitted, we proposed to prepare ODNs with pendant moieties that catalytically cleave the hybridized target nucleic acid. We discovered, however, a lead which we have called an "oligozyme". This agent, which is an oligonucleotide with nucleoside 4 in the middle of the sequence, was found to be capable of cleaving a complementary target oligonucleotide in a sequence-specific manner. Such an agent would be of immense value if it were able to cleave the *Leishmania* mRNA spliced leader in a catalytic manner. The cleavage efficiency of the ODN with 4 in it was too low, however, to be of therapeutic utility.

With the goal of improving the catalytic cleaving activity of ODNs containing 4, we prepared nucleosides 5 and 6 and incorporated them into ODNs. The rationale behind their design was that the side chains on 4, 5, and 6 bore a weakly acidic

Table IV. Activity of Base-Modified ODNs

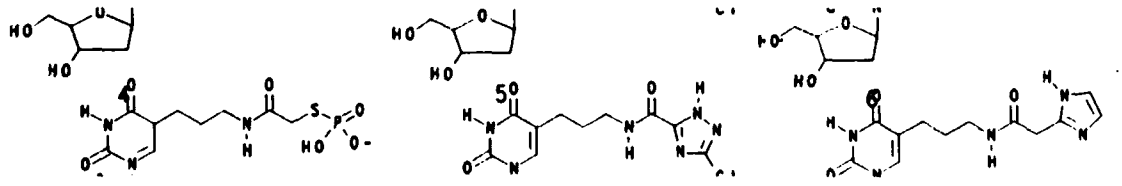
LE 002.01 J	ACT GAT ACT T1T 1TA GCG TT	21
LE 002.02 J	ACT GAT ACT T1T ATA GCG TT	25
LE 002.03 J	ACT GAT ACT T2T ATA GCG TT	22
LE 002.04 J	ACT GAT ACT T2T 2TA GCG TT	27
LE 002.05 J	ACT GAT ACT TAT A3A GCG TT	26
LE 002.06 J	ACT GAT AC3 TAT A3A GCG TT	22
LE 002.07 J	AC3 GAT AC3 TAT A3A GCG 3T	27
LE 001.01 J	CAA TAA AGT ACA GAA AC3 GAT ACT TAT ATA GCG TT	91
LE 001.02 J	CAA TAA AG3 ACA GAA AC3 GAT ACT TAT ATA GCG TT	14
LE 001.04 J	CAA TAA AGT ACA GAA ACT GA4 ACT TAT ATA GCG TT	47
HBV040.01 J	AGG AAT CC3 GAT GTG ATG TTC TCC ATG T3C GTC AC	39

Modifications
1 = Adenosine with 2-acridine
2 = Adenosine with 2'-acridine
3 = deoxyUridine-5-C_{12}
4 = deoxyUridine-5-$(CH_2)_3$-O-$(CH_2)_2$-NHCOCF$_3$

hydrogen which could H-bond with a nucleic acid base on the complementary strand and provide intramolecular general acid catalysis for depurination and cleavage. Tests on these agents showed all to be too ineffective in causing target strand cleavage in a biochemical system to warrent further in vitro testing.

E. Investigate the anti-infective potential of ODNs capable of forming triplexes with DNA.

We feel increasingly strongly about the potential of targeting double-stranded DNA as a means of inhibiting gene expression. The current state of the art in the antisense field does not, however, allow one to predict at this time whether an anti-RNA or anti-DNA strategy is best. Some information is available regarding oligonucleotides that bind to double stranded DNA. Aside from the issues of stability



and delivery that confront any oligodeoxynucleotide (ODN) for use in therapeutics, ODNs that form triple strands with duplex DNA have three serious shortcomings: 1) the third strand (generally all pyrimidines) only binds to purines, giving a "code" of only two letters, 2) the third strand appears to bind rather weakly to the duplex, and

3) the cytosines in the third strand, which bind to guanines in the duplex, must be protonated to properly base pair. Because of the latter, triplexes are unstable at pH values of about 6.6 and higher.

We are investigated the third of these problems by devising a method to eliminate the pH-dependency currently seen in triplex-forming ODNs. We replaced the cytosines in the ODN with a heterocyclic base capable of Hoogsteen bonding to guanine in the DNA duplex without being protonated. This nucleoside was a 5-membered triazole ring identical to cytosine except for the removal of one atom, giving a tautomer preferred for Hoogsteen pairing, as shown in Fig 4. When incorporated into potential triplex forming oligonucleotides, however, this new nucleoside failed to show significant triplex formation in biochemical experiments.

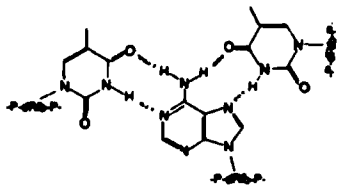


Figure 6. Structure of the T-AT triplex.

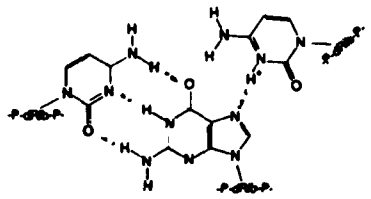


Figure 5. Structure of the C^+-GC triplex.

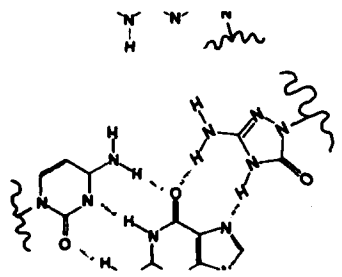


Figure 7. Triplex between new triazole nucleoside and a C-G base pair.

F. Conclusions

We have been unsuccessful in showing that the antisense oligonucleotides that we prepared in this project were able to kill Leishmania in culture. Many novel modifications have been made to the oligos to improve their activity, but none were successful. This is perhaps because of the rapid degradation of these compounds by the parasites. At these concentrations, and with these modifications, an antisense effect can be observed in most other gene expression systems which have been examined. We can only conclude that the Leishmania are refractory to this approach.

1. Toulme, J. J., Krisch, H. M., Loreau, N., Thuong, N. T., and Helene, C. (1986) *Proc. Natl. Acad. Sci. USA* **83**:1227-1231.

2. Verspieren, P., Cornelissen, A.W.C.A., Thuong, N.T., Helene, C., and Toulme, J.J. *Gene* **1987**, *61*, 307-315.

3. Gamper, H.B.,Reed, M. W., Cox, ,Virasco,J. S., Adams,. Gall, A. A,Scholler, J. K., and R. B. Meyer, Jr. *Nucleic Acids Research*,**1993**:21,145-150.